J. Mycopathol. Res. **35**(2) :99-105 (1997) *Printed in India*

Studies on *in vitro* antagonism of some bacterial isolates against *Sclerotium rolfsii* Sacc. causing foot rot of groundnut and sugarbeet

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Out of 68 isolates of microorganisms screened only 11 were primarily selected and found to be inhibitory in different degrees towards 5 soil borne fungal plant pathogens viz., *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium udum*. Among them isolates S_{12} and S_{17} showed high (+++) antagonistic properties followed by isolates S_{21} and S_{16} (++) against *S. rolfsii*. Among these four, isolates S_{12}, S_{17} and S_{11} were collected from the rhizosphere soil of groundnut while isolate S_{16} was obtained from left over and contaminated fungal plates under laboratory condition. Characteristically all these isolates belong to the genus *Bacillus*. Their identity was subsequently confirmed from CMI, Surry, Kew, U.K. and references were given as follows : (i) S_{12} - *Bacillus*, CMI No. 349545 (ii) S_{17} - *Bacillus*, CMI No. 349546 (iii) S_{11} - *Bacillus*, CMI No. 349549 and (iv) S_{16} - *Bacillus*, CMI No. 349548.

Key words : *Bacillus*, biocontrol, *Sclerotium rolfsii*

INTRODUCTION

Sclerotium rolfsii Sacc. the incitant of foot rot of groundnut and sugarbeet in recent years causes considerable damage resulting in loss of production under favourable conditions. Chemical control in one hand has become more expensive and hazardous while breeding of resistant varieties is considered a long term procedure. Biological control, therefore, has thus become more popular world wide (Weller, 1988) mainly due to its non-hazardous effect to nearby ecosystems. The primary necessity is to select potential biocontrol agent through vigorous screening both under laboratory and field conditions. In this aspects, a large number of *Bacillus* spp. have earlier been reported to possess inhibitory properties against a number of plant pathogens (Agarwal *et al.*, 1978; Hedge *et al.*, 1980, Filippi *et al.*, 1987; EL-Kasim *et al.*, 1991; Fiddaman and Rossall, 1993). In the present experiment an *in vitro* study was made following different techniques to explore further the antagonistic properties of some isolates of *Bacillus* spp. against *S rolfsii*.

MATERIALS AND METHODS

The isolates viz. S_{11}, S_{12} and S_{17} were collected from rhizosphere soil of groundnut crop in Nadia district of West Bengal and S^{16} was obtained from contaminated fungal culture under laboratory condition (Ray, 1994).

Serial soil dilution plating was made following the technique described by Johnson and Curl (1972) using soil-extract agar medium (Bunt and Rovira, 1955). Purification of the bacterial isolates were made by streaking and restreaking on agar plates, till a single celled colony was obtained. The cultures were finally maintained in PDA/NA slants at 5°C for future use. *S. rolfsii* was isolated from infected groundnut plants (C.V. - J.L. - 24). For establishment of suppressive effect of the isolated bacteria, several *in vitro* techniques were used against the target pathogen as described below.

(a) Inhibition of growth of host pathogen through cross inoculation studies

The active mycelial plugs of *S. rolfsii* were placed aseptically on opposite side of the PDA plates and allowed to grow for 48 h at 30° ±1°C. The bacterial cultures of 48 h old were then streaked along the straight line in between growth of the fungus and the plates were reincubated at 30° ± 1°C for 5-7 days. The plates were subsequently examined to record any inhibition in growth of the pathogen.

(b) Inhibition zone technique using bacterial plug inoculation

Potato - dextrose - agar at 45°C was seeded with 1.0 ml of bacterial suspension, poured into sterilised Petriplates and allowed to solidify. These plates were incubated for 48 h at 30 ± 1°C.

Small plugs were cut with a disc cutter from the bacterial culture and placed centrally on Petriplates containing PDA. These plates were subsequently inoculated with 6-7 days old fungal plugs surrounding the bacterial plug. This dual cultures were incubated at 30° + 1°C for 6-7 days to observe the inhibition zone, if any.

(c) Growth inhibition of the pathogen by culture filtrates of antagonist bacteria

Cell free culture filtrates from 48 h old PD (Potato - dextrose) broth of the bacterial isolates were prepared by centrifugation at 10,000 G for 15 minutes. Culture filtrate of each antagonistic bacterial isolate (S_{12}, S_{17}, S_{16} and S_{11}) was divided into two parts. One part was autoclaved for 15 minutes and the second part was left unautoclaved. Both autoclaved and unautoclaved parts were diluted with sterilised distilled water and 4 such dilutions viz., 25%, 50%, 75% and 100%, were made. Distilled water either sterilised or nonsterilised served as control. Five to six days old PDA - grown fungal (*S. rolfsii*) plugs were dipped into each inhibitor solution seperately for 2-3 minutes. The fungal plugs were subsequently placed centrally on sterilised PDA Petriplates and incubated at 30° ± 1°C. After 6-7 days, mycelial growth of the fungus was recorded for any inhibition. The entire experiment was conducted under aseptic condition.

(d) Inhibition in germination of sclerotia

PDA plates inoculated with the test fungus were incubated at 30° ± 1°C for 15 days for production of sclerotia. These sclerotia were harvested with a sterilised twiser, air dried for two days, surface sterilised with 0.5% sodium hypochlorite solution, dried in folds of sterilised blotters and stored at 5°C in stoppered conical flasks for future use. Freshly harvested mature sclerotia, apparently of uniform size, colour and age were selected. Seventy two hours old bacterial broth (PD) was centrifused as before and the supernatant was filtered through a milipore filter. One part of this culture filtrate S_{12}, S_{17}, S_{16} and S_{11} was autoclaved and another part was left unautoclaved.

The sclerotia were then dipped into either autoclaved and unautoclaved culture filtrates for 5, 15, 30 minutes, 1 h, 2 h and 3 h. One hundred sclerotia were used for each treatment per culture filtrate for each isolate. The treated sclerotia were placed on previously autoclaved wheat grains placed on moist blotters in sterilised Petriplates. Necessary replications and control were maintained. The Petriplates were incubated at 30°±1°C for 10 days. Observations were recorded at 2 days interval.

(e) Inhibition of pathogen with culture filtrates by agar diffusion technique

Both autoclaved and unautoclaved culture filtrates of each isolate were taken in this study. Sterilised fish-spines were dipped into culture filtrates (autoclaved and unautoclaved) of each bacterium and placed centrally on PDA-Petriplates. Six days old fungal plugs (3 plugs/plate) of *S. rolfsii* were placed surrounding the treated fish spine on Petriplates. The plates were incubated at 30° ± 1°C for 7 days.

(f) Inhibitory effect of culture filtrates through intoxication of medium

Freshly prepared autoclaved and unautoclaved culture filtrates of 4 isolates (S_{17}, S_{12}, S_{11} and S_{16}) were made following methods described earlier. Cool, melted PDA medium were poured in sterilised Petriplates aseptically and was allowed to gel. The culture filtrates (both autoclaved and unautoclaved with respective controls with sterilised and non-sterilised distilled water) were seperately poured in each Petriplates @1.5 ml/plate with sterilised pipette. The culture filtrates were covered with a thin layer of cool, melted PDA medium and allowed to solidify. Six days old fungal *(S. rolfsii)* plug was placed centrally of the PDA-seeded plates and incubated at 30° ± 1°C for 10 days.

(g) Inhibition zone with chloroform extracted culture filtrates using fish-spine technique

The culture filtrates of isolates S_{12} and S'_{17} were taken only for this study. Culture filtrates (autoclaved and unautoclaved) of inhibitory bacteria were mixed thoroughly with 5% chloroform in a separating funnel. The chloroform extracts were separated and sterilised fish-spines were dipped into each category of these chloroform extracts of culture filtrate and placed centrally on PDA seeded sterilised petriplates. Fungal plugs of 6 day age were placed surrounding the treated fish-spine following the earlier procedure.

RESULTS AND DISCUSSION

Using *Sclerotium rolfsii* as test fungus, different methods were employed to study in detail inhibition by some antagonistic bacterial isolates. For this particular pathogen isolates S_{12} and S_{17} showed highest (+++) inhibition and S_{11} and S_{16} the medium (++) under cross inoculation study (Table 1). Using bacterial plugs inoculation technique similar trend in inhibition was recorded and depicted through photographs.

Table 1. *In-vitro* study of growth inhibition by inhibitory bacteria

Bacterial isolates	*S. rolfsii*
S_{12}	+++
S_{17}	+++
S_{11}	++
S_{16}	++
Control	-

`-' = No inhibition; `+' = Poor inhibition; `++' = Moderate inhibition; `+++' = High inhibition.

Table 2. *In-vitro* sensitivity of *S. rolfsii* against culture filtrate of inhibitor bacteria

Bacterial isolates	Unautoclaved culture filtrate					Autoclaved culture filtrate				
	100%	75%	50%	25%	Control	100%	75%	50%	25%	Control
S_{17}	+++	+++	+++	+++	-	-	-	-	-	-
S_{12}	+++	+++	+++	+++	-	-	-	-	-	-
S_{16}	+++	+++	++	+	-	-	-	-	-	-
S_{11}	++	++	++	-	-	-	-	-	-	-

`-' = Not sensitive (full growth); `+' = Mild sensitive (High growth);
`++' = Moderate sensitive (Moderate growth); `+++' = Highly sensitive (Poor growth).

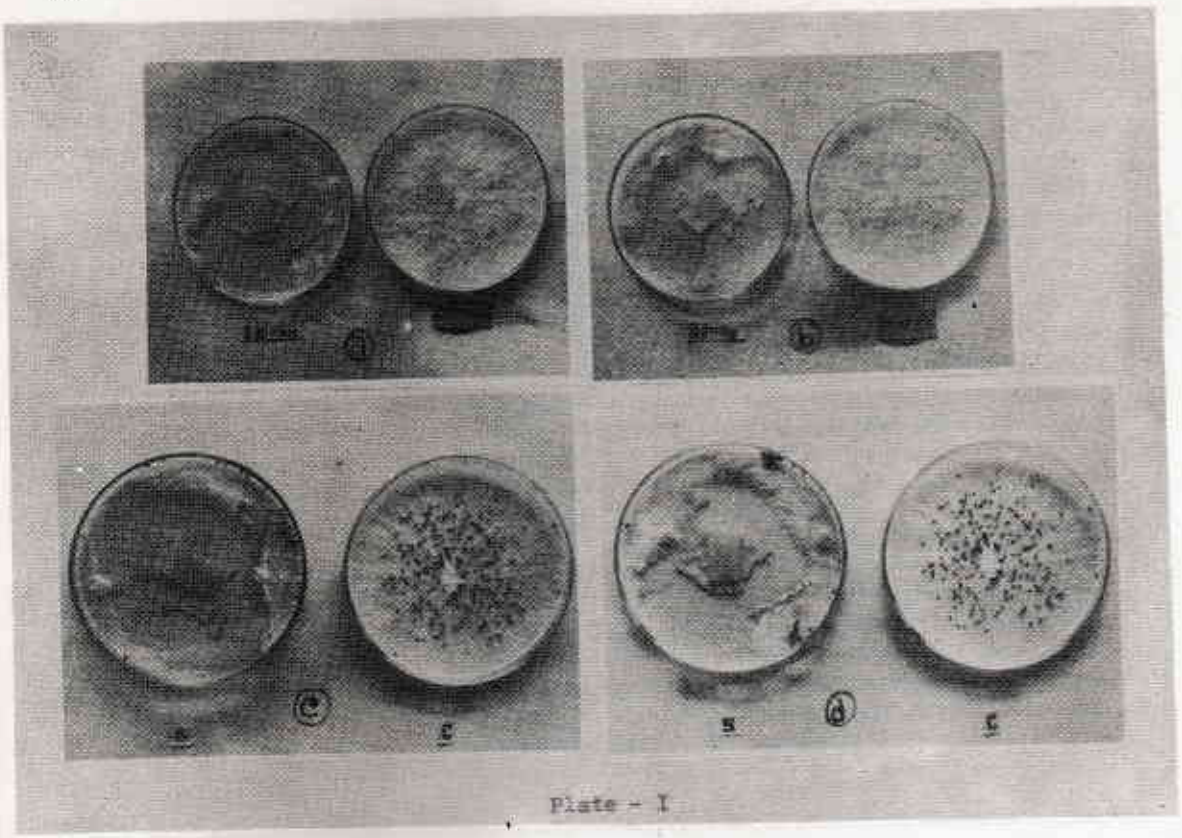

Plate-1 : Inhibition of *S. rolfsii* through bacterial plug inoculation technique : Showing inhibition of mycelial growth of host pathogen by different inhibitors with respectives controls. (a) S_{12} (b) S_{17} (c) S_{16} (d) S_{11}

Growth inhibition of the fungus by culture filtrates of the antagonists in Table 2 showed that inhibition by isolates S_{12} and S_{17} was highest (+++) at all the tested concentrations of unautoclaved culture filtrates. Isolate S_{16} gave highest (+++) inhibition at 100% and 75%, but moderate (++) and poor (+) at 50% and 25% concentrations respectively. Isolate S_{11} exhibited moderate (++) inhibition at three concentrations (100%, 75% and 50%) of unautoclaved culture filtrates. None of the isolates showed any inhibition on the fungal growth when autoclaved culture filtrates were used. Dormant sclerotia are one of the important source of infection on different hosts in fields. Thus, their sensitivity with respect to germination and extent of growth in culture filtrates of inhibitors were examined. Observation from Table 3 revealed that autoclaved culture filtrates failed to inhibit the sclerotial germination and mycelial growth even after different time of treatment. Effect of unautoclaved culture filtrates were insignificant upto 60 minutes of treatment. Effective inhibition of sclerotial germination and in mycelial growth was observed starting from 1 h to 3 h of treatment. Maximum inhibition of both sclerotial germination and mycelial growth was recorded in the range of 0% (-), 2% (+), 10% (+) and 11% (+) after 3 h of treatments with culture filtrates of S_{12}, S_{17}, S_{16} and S_{1} respectively. Inhibition study by agar diffusion technique clearly indicated that all the unautoclaved culture filtrates showed zone of inhibition against the terget pathogen (Table 4).

Table 3. *In vitro* germination of sclerotia and growth of *S. rolfsii* in culture filtrates of inhibitory bacteria after different time of treatment

Time (dipping) in mins	Germination of sclerotia (%) and mycelial growth									
	Isolates									
	S17		S12		S11		S16		Control	
	NS	S	NS	S	NS	S	NS	S	NS	S
5	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++
15	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++	100 +++
30	89 ++	100 +++	86 ++	100 +++	95 ++	100 +++	90 ++	100 +++	100 +++	100 +++
60	30 +	100 +++	20 ++	100 +++	47 +	100 +++	32 +	100 +++	100 +++	100 +++
120	6 +	100 +++	4 +	100 +++	21 +	100 +++	12 +	100 +++	100 +++	100 +++
180	2 +	100 +++	0 -	100 +++	11 +	100 +++	10 +	100 +++	100 +++	100 +++

`-' = No growth; `+' = Poor growth; `++' = Moderate growth; `+++' = Full/good growth; NS = Non sterilised culture filtrate; S = Sterilised culture filtrate.

Table 4. Mycelial growth of *S. rolfsii* near fish-spine dipped in culture filtrates of inhibitory bacteria

Bacterial isolates	Non autoclaved culture filtrate	Autoclaved culture filtrate
S_{17}	16.3*	0.0
S_{12}	14.5	0.0
S_{16}	13.5	0.0
S_{11}	13.3	0.0
Control	0.0	0.0

* Inhibition zone measured in mm.

Among them, isolates S_{17} showed best inhibition (16.3 mm) followed in descending order by S_{12} (14.5 mm), S_{16} (13.5 mm) and S_{11} (13.3 mm). Inhibition in growth was not observed in autoclaved culture filtrates. Complete inhibitory effect on the pathogen by the toxicity of bacterial culture filtrates (Table 5) showed that isolates S_{17} and S_{12} were strong inhibitors when the culture filtrates were unautoclaved. Poor (+) and moderate (++) mycelial growth of the target pathogen was recorded on culture filtrates of isolates S_{16} and S_{11} respectively thus confirming their lower inhibitory properties. It was observed from (Table 6) that both S_{12}

Table 5. *In-vitro* study of culture filtrates of inhibitor bacteria against mycelial growth of *S. rolfsii*

Isolates	Unautoclaved culture filtrate	Autoclaved culture filtrate
S_{17}	-	+++
S_{12}	-	+++
S_{16}	+	+++
S_{11}	++	+++
Control	+++	+++

`-' = No growth of fungus; `+' = Mild growth of fungus; `++' = Moderate growth of gungus; `+++' = Full growth of fungus.

Table 6. *In-vitro* study of chloroform extracted toxic metabolibe from culture filtrates of inhibitory bacteria against mycelium growth of *S. rolfsii*.

Culture filtrate	Unautoclaved culture filtrate + Chloroform	Autoclaved culture filtrate + chloroform
S_{12}	20.85 mm	-
S_{17}	17.85 mm	-
Control	-	-

`-' = No inhibition.

and S_{17} showed good inhibition zone of 20.85 mm and 17.15 mm respectively to check the mycelial growth when treated with chloroform extracted unautoclaved culture filtrates. Biocontrol assay of the selected isolates clearly indicated that inhibition was evident irrespective of the different methods used, like cross inoculation technique, bacterial plug inoculation method, or inhibition tests with culture filtrates of inhibitors in different concentrations, sclerotial germination inhibition test, fish-spine method with unautoclaved and chloroform extracted culture filtrates. Generally the inhibitor principles were thermolabile. The sclerotia of the pathogen became prone to the toxic effect of the culture filtrates requiring at least three hours dip probably requiring sufficient absorption to take place. In this respect isolates S_{12} and S_{17} clearly performed better. A large number of reports of inhibitory effect of *Bacillus* towards plant pathogens including *S rolfsii* are available. Agarwal *et al.* (1978) reported inhibition of *S. rolfsii* by *Bacillus subtilis* with culture filtrate on PDA medium. Hedge *et al.* (1980). Neweigy *et al.* (1982). Shigemitsu *et al.* (1983). Keyser and Ferreira (1988) and others studied different aspects of inhibition of pathogen *in vitro*. However, the present study through the use of different standard methods confirmed beyond doubt the inhibitory principles of the selected isolates against *S. rolfsii*. Persuing biocontrol of a crop disease in field with toxic metabolite, however, need a more rigorous testing before being applied.

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(Accepted for publication 8 May 1997)